Effect of Dietary Supplementation with Vitamin D Metabolites in an Experimental Model of Turkey Osteomyelitis Complex[1]

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ABSTRACT Supplementation with vitamin D_3 was previously shown to protect *Escherichia coli* challenged birds that underwent two dexamethasone (DEX) treatments at 5 and 12 wk of age in an experimental model of turkey osteomyelitis complex (TOC). The purpose of the present study was to determine the effects of dietary supplementation with 10 μg of 1,25 dihydroxyvitamin D_3 (1,25D)/kg feed or 99 μg of 25-hydroxyvitamin D_3 (25D)/kg feed on disease resistance in the same model. Birds were fed the supplemented diets continuously and ad libitum. Seven hundred twenty turkey poults were placed into 24 floor pens in a $3 \times 2 \times 2$ design (three vitamin D treatments, two DEX treatments, two *E. coli* treatments, with two replicate pens per treatment). At 5 wk of age, half of the birds were treated with DEX, and half of the DEX-treated birds and half of the nontreated birds were challenged with *E. coli*. All mortalities and lame birds were necropsied. At 9 wk, all of the DEX- or *E. coli*-treated birds were given another series of DEX injections; 2 wk later 10 birds per pen were necropsied. At 12 wk, survivors of the previous challenges were given a third DEX treatment, and all birds were necropsied 2 wk later. After the first series of DEX injections, mortality was increased in the 25D-supplemented birds that were given the DEX treatment and the *E. coli* challenge. After the second series of DEX injections, the main effect mean BW was significantly lower in birds given 1,25D as compared to controls and 25D-supplemented birds. Mortality was higher in 1,25D-supplemented birds that were challenged with *E. coli* at 5 wk and treated with DEX at 9 wk as compared to 25D-supplemented birds. The 1,25D-treated birds that were treated with DEX at 5 and 9 wk and challenged with *E. coli* at 5 wk had higher mortality and air sacculitis scores as compared to controls and 25D-treated birds. The main effect mean mortality was significantly higher in birds given 1,25D as compared to controls and 25D-treated birds. The percentage of birds with TOC lesions was decreased from 27% to 0 by 25D and 1,25D in the groups given two DEX treatments and *E. coli* challenge. After the third DEX treatment, BW of 1,25D-suppplemented birds was decreased, and mortality and air sacculitis scores were increased. Bone strength was generally increased by supplementation with 1,25D, whereas 25D supplementation increased bone strength only in birds challenged at 5 wk and treated with DEX at Weeks 9 and 12. In this study, supplementation with vitamin D metabolites decreased TOC incidence in *E. coli*-challenged birds given two DEX treatments. However, toxic effects were observed in most supplemented DEX-treated birds and may be attributed to an additive effect of DEX treatment, *E. coli* septicemia, and vitamin D supplementation.

(*Key words*: 1,25 dihydroxyvitamin D_3, 25 hydroxyvitamin D_3, dexamethasone, *Escherichia coli*, turkey)

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INTRODUCTION

Turkey osteomyelitis complex (TOC) is a disease that affects commercially processed turkeys, which can outwardly appear healthy and wholesome yet contain hidden lesions including arthritis and synovitis, abscesses in the soft tissues of the leg and breast, and osteomyelitis of the proximal tibia. This disease has come to the attention of the Food Safety Inspection Service (FSIS) because of the high incidence of *Staphylococcus aureus* isolations from the lesions. Diseased turkeys are currently detected by a federally mandated inspection program that relies on the presence of a green liver to indicate affected carcasses (Cook,

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Abbreviation Key: DEX = dexamethasone; FSIS = Food Safety Inspection Service; IL = interleukin; IFN = interferon; TD = tibial dyschondroplasia; Th = T-helper; TOC = turkey osteomyelitis complex; TPB = tryptose phosphate broth; 1,25D = 1,25 dihydroxyvitamin D_3 ; 25D = 25-hydroxyvitamin D_3.

1988). To understand the etiology of this syndrome, we have developed an experimental model for this disease. This model reproduces all of the lesions of TOC by immunosuppressing 5-wk-old turkeys with a synthetic glucocorticoid, dexamethasone (DEX), followed by challenge with air sac inoculation of 50 to 100 cfu of *Escherichia coli* (Huff et al., 1998, 1999b, 2000). Repeated DEX treatment of survivors results in increased disease incidence. We previously reported that supplementation with vitamin D_3 (cholecalciferol) at times of stress protected birds that underwent two DEX treatments at 5 and 12 wk of age. There was no difference in TOC incidence in birds that underwent only a single DEX treatment at 5 wk of age (Huff et al., 2000). Vitamin D_3 is considered to be biologically inactive, and its in vivo effects are attributed to its various metabolites (DeLuca, 1988; Crowle and Ross, 1990). The most active metabolite is 1,25 dihydroxyvitamin D_3 (1,25D), which has been shown to have wide ranging immunoregulatory and immunomodulatory activity and to directly affect development of all cells of the mononuclear lineage (Ohsugi et al., 1985; Reinhardt and Hustmyer, 1987; Rigby, 1988; Manolagas et al., 1989; Muller and Bendtzen, 1996). Another metabolite, 25-hydroxyvitamin D_3 (25D), has been shown to be advantageous in poultry production (Soares et al., 1995) and is commercially available. The purpose of this study is to determine the effects of continuous feeding of supplemental levels of these two vitamin D metabolites on disease resistance in our TOC model.

MATERIALS AND METHODS

Experimental Design

Seven hundred twenty male Nicholas turkey poults were obtained at 1 d of age, were neck-banded, weighed, and placed into 24 duplicated floor pens in a 3 × 2 × 2 experimental design (3 vitamin D treatments, 2 DEX treatments, 2 *E. coli* treatments) comparing each supplemental vitamin D metabolite treatment to a control diet that contained only the level of vitamin D_3 in the vitamin premix[3] of a standard corn and soybean turkey starter diet (2,860 IU/kg). This diet met or exceeded NRC (1994) recommended levels for all other nutrients. The 1,25D was obtained from the laboratory of R. L. Horst[4] and was added to the base feed at the level of 10 μg/kg. The commercial 25D product (Hy·D[5]) was kindly donated by Roche Vitamins, Inc. The product was a granular premix added at 90 mg/ton (99 μg/kg), as recommended by the manufacturer. Control feed and 25D-supplemented feed also contained an equivalent amount of the vegetable oil that served as diluent for the 1,25D treatment. Birds were fed the supplemented feeds or control feed continuously and ad libitum.

First DEX Treatment (DEX 1)

At 5 wk of age, birds in half of the pens from each treatment were treated with three injections of 2 mg/kg DEX[6] into the thigh muscle on alternating days. DEX concentration was based on mean body weight. On the day of the third DEX injection, half of the DEX-treated birds and half of the nontreated birds were challenged with 50 cfu of *E. coli* in tryptose phosphate broth (TPB) inoculated into the left thoracic air sac. The other half was inoculated with TPB alone. Body weights and feed conversion were determined every other week. Mortality was monitored twice daily after challenge. All dead birds were weighed and examined for lesions of air sacculitis by using the scoring system of Piercy and West (1976) and for TOC lesions by using the FSIS standard 10-cut procedure (Cook, 1988). All TOC lesions and livers were cultured for bacteria on Columbia blood agar, MacConkey agar, and mannitol salt agar. Livers, spleens, heart, and bursae were weighed. Three days after challenge, four birds per pen were bled. Blood was collected for bacterial culture and hematology values, and serum was prepared for clinical chemistry. Total leukocyte counts and the proportions (%) among various leukocytes were determined using a Cell-Dyn 3500 blood analysis system[7] that was standardized for analysis of turkey blood. Heterophil/lymphocyte ratio, an indicator of stress in birds (Gross and Siegel, 1983), was calculated. Clinical chemistry analysis of serum levels of total protein, albumin, glucose, triglycerides, cholesterol, uric acid, and iron, and the enzyme activities of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and creatine kinase were measured with an automated clinical chemistry analyzer.[8]

Serum calcium and magnesium levels were measured by atomic absorption, and serum phosphorus levels were measured with a colorimetric assay. Two weeks after challenge, all birds were evaluated for any signs of lameness, and all lame birds were necropsied and evaluated as described for the mortalities.

Second DEX Treatment (DEX 2)

At 9 wk of age, all DEX-treated birds were treated with another series of three DEX injections. At this time, the birds that had been treated with *E. coli* only were also treated with DEX. Two wk later, five to 10 birds per pen were euthanized, weighed, and necropsied. All mortalities and necropsied birds were evaluated as previously described.

Third DEX Treatment (DEX 3)

At 12 wk of age, surviving DEX-treated birds were again treated with three injections of 2 mg/kg BW DEX on alternate days. Four birds per pen were bled 3 d later, and blood was processed as previously described. All surviving birds were necropsied 2 wk later as previously described. The proximal tibia of each bird was removed for determination of breaking strength by using an Instron Model 4502 Shear

[3]Mitchell Turkey 666, Nutra Blend Corporation, Neosho, MO.
[4]USDA/ARS/NADC, Ames, IA.
[5]Hy-D, Roche Vitamins, Inc., Parsippany, NJ.
[6]Sigma Chemical Co., St. Louis, MO.
[7]Abbott Diagnostics, Abbott Park, IL.
[8]Express Plus, Ciba-Corning Diagnostics Corp., Medfield, MA.

TABLE 1. DEX 1. Effect of supplementation of a standard turkey starter diet with vitamin D in the form of 10 μg/kg 1,25 dihydroxyvitamin D_3 (1,25D) or 99 μg/kg 25 hydroxyvitamin D_3 (25D) on mortality of male turkeys that were untreated (Treatment 1), challenged with air sac inoculation of 50 cfu of *Escherichia coli* at 5 wk (Treatment 2), treated with three injections of 2mg/kg dexamethasone (DEX) at 5 wk (Treatment 3), or treated with DEX and challenged with *E. coli* at 5 wk (Treatment 4) and the main effect mean of vitamin D supplementation over all four treatments

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Main effect mean
	(% Mortality)				
Control feed	0.05 ± 0.22	18.3 ± 39.0	0.00 ± 0.00	48.7 ± 50.3[b]	21.4
25D	0.00 ± 0.00	20.0 ± 40.0	0.03 ± 0.18	65.0 ± 48.0[a]	27.5
1,25D	0.00 ± 0.00	11.7 ± 32.4	0.03 ± 0.18	52.5 ± 50.2[b]	21.4

[a,b]Means in columns with no common superscript are significantly different ($P \leq 0.05$).

Press[9] (Huff et al., 1980). The bones were fat-extracted for 16 h with ethanol in a soxhlet extractor, followed by 16 h with ethyl ether, and ashed at 750 C for 17 h. The right and left proximal tibiae of each bird were scored for incidence and severity of tibial dyschondroplasia (TD) as described by Huff (1980).

Statistics

Data collected from mortalities and from necropsied birds were combined for analysis unless otherwise stated. All percentage data were subjected to arcsine transformation. Pen means were analyzed as a 3 × 2 × 2 factorial arrangement by using the general linear models and least square means procedures of SAS software (SAS Institute, 1988). Significant differences between treatments were separated using Duncan's multiple-range test. $P \leq 0.05$ was considered significant.

RESULTS

Body weights of birds supplemented with 1,25D were significantly higher than controls at 2 wk of age ($P = 0.009$), with a marginal improvement observed with 25D supplementation ($P = 0.06$). There were no differences in BW at 4 wk of age with supplementation of either vitamin D metabolite. Average daily gain was higher in birds treated with either metabolite for the first 2 wk (25D, $P = 0.04$; 1,25D, $P = 0.007$). There were no significant differences in feed conversion at 2 or 4 wk of age (data not shown).

DEX 1

There were no significant differences in BW or feed conversion due to vitamin D metabolite supplementation at 7 wk of age, 2 wk after DEX/*E. coli* challenge (data not shown). There was an increase in mortality in the 25D-supplemented birds that were given the DEX treatment and the *E. coli* challenge (Treatment 4), as compared to both non-supplemented controls and 1,25D-supplemented birds (Table 1). There were no significant effects of vitamin D metabolite supplementation on serum calcium, phosphorus, or magnesium levels; however, birds given Treatment 2 and supplemented with 25D had higher calcium levels than those supplemented with 1,25D (Table 2). DEX treatment had no effect on serum calcium or magnesium. DEX treatment, however, significantly decreased serum phosphorus levels in non-supplemented birds and 1,25D-supplemented birds given Treatment 3 or 4 and in 25D supplemented birds given Treatment 4 (Table 2). Serum levels of glucose were higher in 1,25D birds given Treatment 3 (DEX only) as compared to controls and 25D-supplemented birds. Glucose was higher in all DEX-treated birds (Treatments 3 and 4) as compared to birds not treated with DEX regardless of vitamin D supplementation. Birds supplemented with 1,25D and given Treatment 3 had higher glucose levels than Treatment 4 birds (Table 2). The only significant difference in white blood cell total or differential counts due to vitamin D metabolite supplementation was an increase in the main effect mean percentage of eosinophils in 1,25D-supplemented birds as compared to controls (control = 4.37%; 25D = 6.61%, $P = 0.08$; 1,25D = 6.97%, $P = 0.04$).

DEX 2

Main effect mean body weights were significantly lower in birds given 1,25D as compared to controls and 25D-supplemented birds (Table 3). There were no differences in feed conversion (data not shown). Percentage mortality was significantly higher in 1,25-supplemented birds given Treatment 2 as compared to 25D-supplemented birds and in 1,25D birds given Treatment 3 as compared to controls and 25D birds (Table 3). The main effect mean mortality incidence was significantly higher in birds given 1,25D as compared to controls and 25D birds (Table 3). Air sacculitis scores of Treatment 3 were higher in birds supplemented with 1,25D as compared to controls and 25D birds, and the main effect mean air sacculitis score was higher for 1,25D-supplemented birds as compared to 25D-supplemented birds (Table 3). The percentage of birds with lesions of TOC was significantly decreased in birds supplemented with 25D or 1,25D and given Treatment 4, and the main effect mean for 1,25D was significantly lower than the control (Table 3).

[9]Instron Corp., Canton, MA.

TABLE 2. DEX 1. Effect of supplementation of a standard turkey starter diet with vitamin D in the form of 10 μg/kg 1,25 dihydroxyvitamin D_3 (1,25D) or 99 μg/kg 25 hydroxyvitamin D_3 (25D) on serum calcium, phosphorous, and glucose levels of male turkeys that were untreated (Treatment 1), challenged with air sac inoculation of 50 cfu of *Escherichia coli* at 5 wk (Treatment 2), treated with three injections of 2 mg/kg BW dexamethasone (DEX) at 5 wk (Treatment 3), or treated with DEX and challenged with *E. coli* at 5 wk (Treatment 4) and the main effect mean of vitamin D supplementation for all four treatments

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Main effect mean
Serum calcium (mg/dL)					
Control feed	13.49 ± 0.86	13.92 ± 2.06^{ab}	12.76 ± 1.10	12.56 ± 2.37	13.18
25D	13.56 ± 1.47	15.07 ± 1.91^{a}	15.31 ± 1.83	9.825 ± 1.49	13.32
1,25D	13.03 ± 0.95	10.11 ± 0.98^{b}	11.85 ± 1.39	12.38 ± 1.45	11.80
Serum phosphorus (mg/dL)					
Control feed	13.70 ± 1.05^{x}	11.00 ± 1.40^{xy}	9.54 ± 1.72^{y}	7.33 ± 1.37^{y}	10.70
25D	12.33 ± 1.18^{x}	12.58 ± 2.00^{x}	9.10 ± 1.27^{xy}	8.14 ± 1.19^{y}	10.78
1,25D	13.14 ± 1.43^{x}	11.16 ± 1.37^{xy}	10.08 ± 1.27^{y}	8.03 ± 0.73^{y}	10.66
Serum glucose (mg/dL)					
Control feed	237.75 ± 9.35^{x}	243.00 ± 7.68^{x}	494.88 ± 42.86^{b,y}	429.13 ± 73.38^{y}	351.19
25D	249.14 ± 5.87^{x}	247.88 ± 7.42^{x}	477.63 ± 46.22^{b,y}	405.75 ± 29.21^{y}	348.19
1,25D	241.00 ± 13.24^{x}	229.50 ± 6.61^{x}	609.00 ± 38.50^{a,z}	450.50 ± 39.98^{y}	382.50

^{a,b}Means in columns with no common superscript are significantly different ($P \leq 0.05$).

^{x,y,z}Means in rows with no common superscript are significantly different ($P \leq 0.05$).

DEX 3

Body weights were significantly lower in 1,25D-supplemented birds given Treatment 3 as compared to controls and 25D-supplemented birds and were also lower in 1,25D birds given Treatment 4 as compared to 25D birds (Table 4). The main effect mean BW of 1,25D-supplemented birds was lower than the control and 25D birds. There were no consistent differences in feed conversion (data not shown). Percentage mortality and air sacculitis scores were significantly higher in 25D-supplemented birds given Treatment 3 as compared to controls (Table 4). Bone strength (load to break as measured by shear press) was significantly higher in birds given Treatment 1 and supplemented with 1,25D compared to controls. Both 1,25D and 25D increased bone strength in birds given Treatment 2. Birds supplemented with 1,25D had higher bone strength compared to controls when given Treatment 3 and had a higher main effect mean bone strength as compared to controls (Table 4). The percentage of broken femurs at necropsy was significantly lower in birds given Treatment 3 and supplemented with 1,25D or 25D (both 0%) as compared to controls (12.5%, $P = 0.03$). There were no significant differences in serum calcium, phosphorus, magnesium, or glucose levels after DEX 3 (data not shown). The main effect mean for the percentage of monocytes in peripheral blood was significantly higher in 1,25D-supplemented birds as compared to non-supplemented controls (control = 10.6%, 25D = 11.2%, 1,25D = 12.8%; $P = 0.05$). The percentage of monocytes was significantly higher only in those birds from Treatment 3 (DEX only) that were supplemented with 1,25D (control = 7%, 25D = 12%, 1,25 D = 16%; $P = 0.02$).

TABLE 3. DEX 2. Effect of supplementation of a standard turkey starter diet with vitamin D in the form of 10 μg/kg 1,25 dihydroxyvitamin D_3 (1,25D) or 99 μg/kg 25 hydroxyvitamin D_3 (25D) on BW, percentage mortality, air sacculitis scores, and percentage incidence of turkey osteomyelitis complex of male turkeys that were untreated (Treatment 1), challenged with air sac inoculation of 50 cfu of *Escherichia coli* at 5 wk followed by treatment with three injections of 2mg/kg BW dexamethasone (DEX) at 9 wk (Treatment 2), treated with DEX at 5 wk and again at 9 wk (Treatment 3), or treated with DEX and challenged with *E. coli* at 5 wk, then treated again with DEX at 9 wk (Treatment 4) and the main effect mean of vitamin D supplementation over all four treatments

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Mean effect mean
BW (g)					
Control feed	6,630.0 ± 167.2	4,430.4 ± 163.4	2,945.4 ± 116.0	3,096.4 ± 174.4	4,289.3^{a}
25D	6,711.2 ± 217.0	4,216.0 ± 192.5	3,097.5 ± 126.6	3,135.0 ± 223.7	4,420.0^{a}
1,25D	7,062.1 ± 147.0	4,057.6 ± 127.1	2,777.7 ± 103.8	2,715.0 ± 206.5	4,042.4^{b}
Percentage mortality					
Control feed	0.00 ± 0.00	6.98 ± 3.93^{ab}	7.14 ± 3.47^{b}	9.52 ± 6.56	5.73^{b}
25D	5.41 ± 3.77	0.00 ± 0.00^{b}	7.55 ± 3.66^{b}	0.00 ± 0.00	4.23^{b}
1,25D	0.00 ± 0.00	13.21 ± 4.7^{a}	20.75 ± 5.62^{a}	11.11 ± 6.16	12.28^{a}
Mean air sacculitis score					
Control feed	0.06 ± 0.06	0.74 ± 0.28	0.78 ± 0.35^{b}	0.30 ± 0.21	0.54^{ab}
25D	0.11 ± 0.11	0.28 ± 0.18	0.54 ± 0.26^{b}	0.33 ± 0.33	0.33^{b}
1,25D	0.00 ± 0.00	0.81 ± 0.27	1.47 ± 0.35^{a}	0.71 ± 0.41	0.84^{a}
Percent turkey osteomyelitis complex					
Control feed	0.00 ± 0.00	8.70 ± 6.01	4.17 ± 4.17	27.27 ± 14.08^{b}	4.89^{a}
25D	5.26 ± 5.26	5.00 ± 5.00	0.00 ± 0.00	0.00 ± 0.00^{a}	2.90^{ab}
1,25D	0.00 ± 0.00	3.70 ± 3.70	0.00 ± 0.00	0.00 ± 0.00^{a}	1.01^{b}

^{a,b}Means in columns with no common superscript are significantly different ($P \leq 0.05$).

TABLE 4. DEX 3. Effect of supplementation of a standard turkey starter diet with vitamin D in the form of 10 μg/kg 1,25 dihydroxyvitamin D_3 (1,25D) or 99 μg/kg 25 hydroxyvitamin D_3 (25D) on BW, percentage mortality, air sacculitis scores, and bone strength of male turkeys that were untreated (Treatment 1); challenged with air sac inoculation of 50 cfu of *Escherichia coli* at 5 wk followed by treatment with three injections of 2m/kg BW dexamethasone (DEX) at 9 wk and again at 12 wk (Treatment 2); treated with DEX at 5 wk, 9 wk, and 12 wk (Treatment 3); or treated with DEX and challenged with *E. coli* at 5 wk, then treated again with DEX at 9 wk and 12 wk (Treatment 4) and the main effect mean of vitamin D supplementation for all four treatments

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Main effect mean
BW					
Control feed	11,946.9 ± 205.1	6,557.4 ± 194.0	5,014.2 ± 203.9[a]	5,743.3 ± 314.2[aa]	7,374.8[a]
25D	12,227.2 ± 206.3	6,751.0 ± 260.1	5,319.1 ± 252.3[a]	6,523.3 ± 756.9[a]	7,710.3[a]
1,25D	12,036.3 ± 173.4	6,492.9 ± 257.0	4,364.7 ± 199.9[b]	5,216.4 ± 254.3[b]	7,027.5[b]
Percentage mortality					
Control feed	0.00 ± 0.00	5.26 ± 5.26	16.67 ± 7.77[b]	10.00 ± 10.00	8.33
25D	0.00 ± 0.00	10.00 ± 6.88	39.17 ± 10.41[a]	0.00 ± 0.00	16.42
1,25D	0.00 ± 0.00	0.00 ± 0.00	31.82 ± 10.16[ab]	15.38 ± 10.42	12.00
Air sacculitis score					
Control feed	0.00 ± 0.00	0.29 ± 0.29	0.68 ± 0.28[b]	0.22 ± 0.22	0.33
25D	0.17 ± 0.12	0.37 ± 0.21	1.33 ± 0.31[a]	0.40 ± 0.40	0.63
1,25D	0.00 ± 0.00	0.43 ± 0.25	0.67 ± 0.33[b]	0.23 ± 0.23	0.35
Bone strength					
Control feed	72.4 ± 4.1[b]	44.5 ± 2.5[b]	48.3 ± 3.9[b]	56.7 ± 4.5	55.5[b]
25D	79.0 ± 4.7[ab]	57.8 ± 5.0[a]	51.7 ± 4.2[ab]	53.8 ± 2.6	61.0[ab]
1,25D	89.7 ± 4.0[a]	57.9 ± 4.9[a]	59.4 ± 3.2[a]	56.9 ± 2.9	66.0[a]

[a,b]Means in columns with no common superscript are significantly different ($P \leq 0.05$).

The percentage of eosinophils was higher in 1,25D-supplemented birds of Treatment 4 (DEX/*E. coli*) as compared to controls (control = 0.2%, 25D = 5%, 1,25D = 7%, $P = 0.05$). The main effect mean total red blood cell count and hematocrit were significantly higher in 1,25D-supplemented birds as compared to non-supplemented controls (data not shown). There were no differences in the incidence of tibial dyschondropasia affected by vitamin D supplementation; however, DEX treatment significantly decreased TD incidence and score (data not shown).

DISCUSSION

The purpose of this study was to determine whether supplementation of a standard turkey diet with vitamin D metabolites would decrease disease incidence in an experimental model of TOC. The incidence of TOC lesions was decreased from 27% to 0 by supplementation with 25D or 1,25D in birds at 11 wk of age, after two DEX treatments and *E. coli* challenge. There was no effect on TOC incidence in other treatment groups. This positive result, however, is overshadowed by data that suggest such continuous dietary supplementation with either of these vitamin D metabolites at these levels also resulted in higher mortality rates in birds treated with DEX. It should be emphasized that these negative effects did not occur in control birds that were not treated with DEX. Before challenge all 1,25D-supplemented birds had significantly higher BW at Week 2, and birds given either vitamin D supplement had higher daily gains during the first 2 wk. Supplementation with 1,25D also significantly increased bone strength of 14-wk-old control birds. The safety and efficacy of 25D to promote growth and bone development in poultry have been demonstrated (Cantor and Bacon, 1978; Soares et al., 1995; Yarger et al., 1995a,b; Lanenga et al., 1999; Terry et al., 1999), and the product used in this study is "generally recognized as safe" (GRAS) and has been sold commercially since 1994.

Supplementation with these vitamin D metabolites was not as effective in protecting turkeys in this model as was a commercially available vitamin D_3 (cholecalciferol) product used to supplement drinking water. We have reported previous studies that suggest drinking water supplementation with cholecalciferol for the first 5 d after hatch and again for 12 h before and after each stressful event, including weighing and DEX treatments, resulted in increased resistance to opportunistic bacterial infection resulting from multiple DEX treatments (Huff et al., 2000). Supplemented birds that were challenged with DEX injection at both 5 and 12 wk of age had less mortality, lower air sacculitis scores, and decreased incidence of TOC, green liver, heterophil/lymphocyte ratio, and bacterial isolation from tissues, as well as increased BW, when compared to non-supplemented controls. A major difference between these previous studies and the present study is the use of continuous supplementation in feed rather than intermittent drinking water application of the vitamin. Because 25D and 1,25D are more metabolically active and were supplemented throughout the growing period, and in addition to 2,860 IU/kg cholecalciferol, it is possible that the given levels, although safe in the control birds, might have been toxic to some of the DEX-treated birds. The high super-physiological dosage of DEX used in this study was meant to simulate the stress response of a small proportion of male turkeys that may develop TOC under field conditions. It appears from these data that in birds experiencing severe stress, supplemental vitamin D metabolites may be contra-indicated even though they provide increased growth and improved bone development in the general population.

Mirales et al. (1999) also found a negative relationship between stress and 25D supplementation. They reported that broiler chickens supplemented with 68.9 mg/kg 25D and stressed with a coccidiosis challenge had increased mortality relative to those stressed birds that were not supplemented. These authors suggested that the increase in performance observed with 25D-supplemented birds was due to immunosuppression of the acute phase response and enhancement of the humoral response, which they described as a shift from a T-helper (Th)1 to a Th2 immune response.

In mammals, the Th1 cytokine profile is characterized by an excess of interleukin (IL)2, IL12, and interferon (IFN)γ and results in stimulation of cellular immunity and inflammation, whereas the Th2 cytokine profile is associated with stress situations, excess glucocorticoid, and stimulated humoral responses and is characterized by excess IL4, IL6, and IL10 and deficient IL2, IL12, and IFNγ (Mosmann and Coffman, 1989; Hassig et al., 1996; Spellberg and Edwards, 2001). A similar Th1/Th2 dichotomy has recently been described in chickens (Vandaveer et al., 2001).

A number of physical and psychological stressors, as well as glucocorticoid treatment, have been shown to shift cytokine production from a predominantly Th1 to a Th 2 profile (Agarwal and Marshall, 1998; Elenkov and Chrousos, 1999; Rook, 1999). The very same effects have been attributed to 1,25D (Lemire et al., 1995; Overbergh et al., 2000). The combination of glucocorticoid treatment and vitamin D supplementation observed in the present study might have cooperatively suppressed cell-mediated immunity to a dangerous level. Recently, the suppression of the Th1 immune response by DEX in vitro has been shown to be further enhanced by 1,25D, which was also shown to upregulate Th2 cytokines, and inhibit the suppression of Th2 cytokines by glucocorticoids (Jirapongsananuruk et al., 2000). This additive effect of DEX and 1,25D treatment described for cytokine production in vitro may explain the negative effects of supplementation observed only in the stressed birds of the present study. Animals that are under stress or that are receiving glucocorticoid treatment are primed to develop a Th2 response, even to antigens that would normally elicit a Th1 response (Spellberg and Edwards, 2001). In vivo studies of rats have also shown that DEX remarkably increased mortality due to a sublethal dose of vitamin D_2 (Kunitomo et al., 1989). The increase in eosinophils observed in birds supplemented with vitamin D metabolites after DEX 1 and DEX 3 suggests a toxic response. Eosinophils are increased during allergic disease and other inflammatory diseases and are thought to cause tissue damage and the propagation of inflammation when interacting with Th2 cells and antigen-presenting cells (Elsner and Kapp, 2000). A clear increase of eosinophils was associated with hypervitaminosis of D_3 (Krempl and Bacowsky, 1988; Bacowsky et al., 1988), and chick embryos injected with large doses of 1,25D showed infiltration of spleen, liver, and bursa of Fabricius with eosinophils (Narbaitz, 1987).

1,25D has been shown to down-regulate IL12 expression in macrophages and dendritic cells, providing a mechanism for the shift from a Th1 cytokine profile to a Th2 profile (D'Ambrosio et al., 1998). This metabolite inhibits the growth of *Mycobacteria tuberculosis* in cultured human monocytes and macrophages (Crowle et al., 1987; Crowle and Ross, 1990) and improves resistance to tuberculosis in a murine model (McMurray et al., 1990), suggesting an improvement in bactericidal activity. Macrophages from vitamin D_3-deficient mice function abnormally, and their function can be restored in vitro and in vivo by treatment with 1,25D (Reichel et al., 1985). However, paradoxically, a similar disease, experimental murine paratuberculosis, is exacerbated by vitamin D-induced hypercalcemia (Stabel and Goff, 1996). Several other granulomatous diseases, including sarcoidosis, Crohn's disease, and cat scratch fever (Fuss et al., 1992; Bosch, 1998a,b), cause lesions that produce large quantities of 1,25D and result in hypercalcemia.

Vitamin D has been shown to specifically modulate the deleterious effect of the stress response on macrophage function (Puchacz et al., 1996). The decrease of TOC lesion incidence by vitamin D supplementation in birds challenged with *E. coli* and given three DEX treatments may be due to an enhanced ability of supplemented birds to clear internalized bacteria sequestered but not killed by phagocytic cells. We have previously shown that the immunosuppression induced by DEX results in decreased bactericidal activity of peripheral blood glass-adherent mononuclear cells, and that males are more affected by DEX treatment than females (Huff et al., 1999c). Because TOC is a disease that mainly affects male turkeys, we have hypothesized that defective macrophage function of male turkeys may be involved in its pathogenesis (Huff et al., 1999a).

In studies with broiler chickens, deficiency of vitamin D has been shown to decrease cell-mediated immunity, including thymus weights, cutaneous basophil hypersensitivity response, and phagocytosis by sephadex-elicited abdominal macrophages, with no effect on humoral immunity (Aslam et al., 1998). The addition of 20 μg/kg supplemental 1,25D to turkey diets has been shown to increase antibody response to sheep erythrocytes, increase phagocytosis by sephadex-elicited abdominal macrophages, and increase the percentage of tumor cells killed by lipopolysaccharide-stimulated abdominal macrophages (Garlich et al., 1992). Although it appears that 1,25D can enhance some immune responses and that a deficiency of vitamin D results in increased susceptibility to infection, it should be remembered that this metabolite is effective in depressing the cellular immune response in cases of autoimmune disease and transplantation and is being used clinically to control inflammatory diseases (Lemire et al., 1995). Deficiency and excess of this vitamin can depress cell-mediated immunity, necessitating that a balance be obtained in its use as a dietary supplement.

It appears from these data that cumulative stress is as important as experimental bacterial challenge in this disease model. It can be inferred that repeatedly stressed

birds will become naturally challenged by opportunistic pathogens present in the environment and that susceptibility to infection increases with each exposure to stress. In previous experiments we have observed that in younger birds given multiple DEX treatments or in older birds given a single DEX treatment, bacterial inoculation is often unnecessary for the disease process to occur (Huff et al., 1999b; 2000). The birds are sufficiently immunosuppressed so that exposure to the normal bacterial flora of their environment is sufficient for the development of air sacculitis and TOC lesions.

The increase in bone strength observed in 14-wk-old control birds supplemented with either vitamin D metabolite was also indicated by a decrease in the incidence of broken femurs at necropsy. The turkeys in this experiment were maintained in blacked-out houses under incandescent light for 23 h per day. This environment, although common in commercial turkey production in some regions, in effect eliminated vitamin D synthesis from exposure to ultraviolet light and might have also affected immune responses through disruption of circadian rhythm (Kirby and Froman, 1991). Appropriate supplementation with vitamin D to maximize bone physiology and enhance immune response presents a dilemma. The vitamin must be supplied in the diet, but because it can be destroyed by oxidation and its utilization can be inhibited by mycotoxins, it has been reported that even diets supplemented with excess can be deficient (NRC, 1994). On the other hand, high levels of the vitamin can cause bone calcium loss resulting in hypercalcemia and immunosuppression as well as leg problems (Cruikshank and Sim, 1987; Bacowsky et al., 1988). In several studies, 1,25D has been shown to decrease the incidence of TD (Edwards, 1992); however, in this study there was no effect of vitamin D supplementation on TD incidence or severity. Treatment with DEX did significantly reduce TD incidence; however, this effect was probably due to the decrease in BW caused by DEX treatment. It was surprising that supplementation with vitamin D metabolites did not affect serum levels of calcium or phosphorus, but there could have been an effect on the activity of 1,25D in the gut or bone. The deleterious effects of DEX treatment on serum phosphorus and glucose levels are consistent with reported effects of glucocorticoid treatment in mammals and were not alleviated by vitamin D treatment.

In summary, supplementation of a complete turkey diet with 25D and 1,25D decreased the incidence of TOC after birds were challenged with two DEX treatments and *E. coli*; however, signs of vitamin D toxicity, including increased mortality and eosinophilia were also observed with some DEX treatments. Because glucocorticoid treatment, *E. coli* septicemia, and supplementation with 1,25D have all been associated with a shift from a predominantly Th1 to a Th2 cytokine response, the treatment of stressed birds with vitamin D metabolites may exacerbate some disease situations by dangerously decreasing the inflammatory response to infection.

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